Biochimica et Biophysica Acta 1834 (2013) 1352–1358

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Review

Chemical proteomics and functional proteomics strategies for protein kinase inhibitor validation and protein kinase substrate identification: Applications to protein kinase CK2☆

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ARTICLE INFO

Article history:
Received 14 January 2013
Accepted 4 February 2013
Available online 14 February 2013

Keywords:
CK2
Protein kinase inhibitor
TBB
Chemoproteomics
Functional proteomics
Inhibitor profiling

ABSTRACT

Since protein kinases have been implicated in numerous human diseases, kinase inhibitors have emerged as promising therapeutic agents. Despite this promise, there has been a relative lag in the development of unbiased strategies to validate both inhibitor specificity and the ability to inhibit target activity within living cells. To overcome these limitations, our efforts have been focused on the development of systematic strategies that employ chemical and functional proteomics. We utilized these strategies to evaluate small molecule inhibitors of protein kinase CK2, a constitutively active kinase that has recently emerged as target for anti-cancer therapy in clinical trials. Our chemical proteomics strategies used ATP or CK2 inhibitors immobilized on sepharose beads together with mass spectrometry to capture and identify binding partners from cell extracts. These studies have verified that interactions between CK2 and its inhibitors occur in complex mixtures. However, in the case of CK2 inhibitors related to 4,5,6,7-tetrabromo-1H-benzotriazole (TBB), our work has also revealed off-targets for the inhibitors. To complement these studies, we devised functional proteomics approaches to identify proteins that exhibit decreases in phosphorylation when cells are treated with CK2 inhibitors. To identify and validate those proteins that are direct substrates for CK2, we have also employed mutants of CK2 with decreased inhibitor sensitivity. Overall, our studies have yielded systematic platforms for studying CK2 inhibitors which we believe will foster efforts to define the biological functions of CK2 and to rigorously investigate its potential as a candidate for molecular-targeted therapy. This article is part of a Special Issue entitled: Inhibitors of Protein Kinases (2012).

1. Introduction

Protein kinases play universal roles in the regulation of many biological processes and have been implicated in a large number of human diseases including many forms of malignancy [1,2]. In fact, a recent analysis of available genomics data revealed nearly one thousand kinase mutations in ~70 single-gene disorders, the majority of which (50) are associated with cancer [3]. With the ongoing explosion of genome sequencing, it is anticipated that the number of kinase mutations associated with cancer and other diseases will continue to mount [4,5]. The remarkable success of Gleevec/imatinib, the first protein tyrosine kinase inhibitor drug to receive FDA approval, for the treatment of Chronic Myelogenous Leukemia (CML) was a seminal advance demonstrating the utility of kinase inhibitors as therapeutic agents [6,7]. This revelation that kinases are "druggable" together with the striking prevalence of kinase alterations in disease has spurred intense interest in the development of kinase inhibitor drugs [2,5,8]. Furthermore, the universal involvement of phosphorylation as a regulatory mechanism has resulted in widespread use of protein kinase inhibitors as agents for interrogating the involvement of protein kinases in a vast array of experimental systems.

Despite their promise as therapeutic agents and routine use in experimental studies, a number of significant challenges arise both in the design and in the utilization of protein kinase inhibitors. First of all, protein kinases represent a large family comprising more than 500 members within the human genome. Consequently, the conserved architecture and shared catalytic machinery of these enzymes present major hurdles in the development of specific inhibitors, especially for ATP-competitive kinase inhibitors that continue to represent the majority of kinase inhibitors which are currently available. It is also evident that in many instances, the inhibitory characteristics of protein kinase inhibitors are primarily derived from studies performed with purified, often recombinant, components that may not accurately reflect physiological conditions within living cells. Overall, while protein kinase inhibitors offer

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1570-9639/$ – see front matter
http://dx.doi.org/10.1016/j.bbapap.2013.02.006

tremendous promise both as research tools and as potential therapeutic agents, limitations with their use are well documented [1,9–12].

Based on our own experiences with protein kinase inhibitors, we have devised a combination of functional proteomics and chemical proteomics strategies to address two major issues related to our use of protein kinase inhibitors for experimental studies. The first issue (described in the next section) relates to the development of strategies to demonstrate that the kinase inhibitor did in fact engage its intended target within cells (i.e., how do we know that the inhibitor worked to inhibit its intended target?) [13]. The second issue (described in Sections 2 and 3) relates to the systematic and unbiased analysis of inhibitors to further evaluate its interactions with both its intended target and any potential off-targets (i.e., what else interacts with the inhibitor in complex mixtures?) [14–16]. Although the studies that we have reviewed were focused on an evaluation of inhibitors for protein kinase CK2, we expect that the approaches that we have devised can be readily adapted to other protein kinase inhibitors.

2. On-target inhibition: Kinase inhibitor validation

A critical step in drug validation is demonstrating that the drug engages its intended target within living cells. In the case of protein kinase inhibitors, it is important to identify proteins that are direct substrates of the kinase(s) that are the intended targets of the inhibitor with the expectation that target engagement within living cells would result in inhibitor-dependent decreases in phosphorylation of these kinase substrates. Although there are a vast number of published reports that exploit protein kinase inhibitors to investigate the roles of protein kinases in biological processes and to explore their potential utility as therapeutic agents [17–20], a surprising number of these reports fail to demonstrate inhibitor-dependent changes in phosphorylation of kinase substrates [13]. Even heavily studied kinases with widely available inhibitors can be subject to this limitation [17]. A case in point is protein kinase CK2, a small family of constitutively active enzymes that has recently emerged as a candidate for cancer therapy [21–25]. Hundreds of potential CK2 substrates have been described, often on the basis of CK2 consensus recognition motifs within phosphopeptides that are identified in phosphoproteomics studies [26]. Furthermore, many CK2 inhibitors have been developed including a number of compounds that are commercially available and have been widely utilized [18–20,25,27–32]. Despite these advances, when using CK2 inhibitors it has generally remained challenging to assess whether the inhibitor actually attenuated CK2 activity in cells. To overcome this limitation, we have devised a functional proteomics strategy that involves the identification of proteins that exhibit inhibitor-dependent decreases in phosphorylation (Fig. 1). Furthermore, to ensure that these proteins are direct substrates of CK2, we have exploited mutants of CK2 with decreased sensitivity to the inhibitors to perform rescue experiments both using recombinant proteins and inhibitor-treated cells [13].

To use our functional proteomic kinase inhibitor on-target identification and validation strategy (Fig. 1) we labeled cells with radioactive orthophosphate in combination with kinase inhibitor treatment to evaluate global phosphorylation changes in the cellular proteome [13]. Given their widespread representation in the literature, we focused our efforts on commercially available CK2 inhibitors including 4,5,6,7-tetrabromobenzotriazole (TBB, Sigma) [20] and two of its derivatives 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT, Calbiochem) [19], and 4,5,6,7-tetrabromobenzimidazole (TBBz, Sigma) [32]. In our initial studies, inhibitor-dependent changes in phosphate labeling were monitored after 12 h of inhibitor treatment by two dimensional gels and differentially ^{32}P labeled proteins were identified by mass spectrometry. Several of these proteins were identified as candidate CK2 substrates on the basis of their appearance within existing phosphorylation databases (e.g. PhosphoSitePlus® (www.phosphosite.org) [33], Phosida (http://www.phosida.de/) [34] or PhosphoElm http://phospho.elm.eu.org/) [35] and by literature searches (PubMed http://www.ncbi.nlm.nih.gov/pubmed). The following inclusion criteria were used for literature searches: (A) if they were reported as phosphorylated proteins in cells and (B) if they were reported as substrates of CK2 in the literature. Based on these search parameters, we selected eukaryotic elongation factor 1 delta (EEF1D) to carry out our validation strategy because: i) EEF1D was previously shown to be phosphorylated in the cells at multiple residues based on the database and literature search results [36–40], ii) it showed significant reduction in ^{32}P incorporation after the CK2 inhibitor treatments in our assays, and iii) it had not previously been validated as a CK2 substrate by an unbiased strategy [13].

To further evaluate EEF1D as a potential physiological CK2 substrate, we developed a phospho-specific antibody targeting (pSer162) one of its potential CK2 phosphorylation sites. Using these antibodies, decreased phosphorylation of pSer162 was detected, both with EEF1D-FLAG and with endogenous EEF1D following treatment with CK2 inhibitors or siRNA-mediated knockdown of CK2. The CK2 siRNA treatments not only showed that the level of individual CK2 subunits in the cells could be reduced significantly, but also validated that the phosphorylation at pSer162 residue on EEF1D was reduced and that this phosphorylation reduction could be monitored by our phospho-EEF1D antibody [13].

To demonstrate that EEF1D is a direct target of CK2, we developed a method that allowed us not only to confirm that the inhibitor modified the activity of the on-target kinase but also provided a tool to validate the identified protein as a *bona fide* substrate of the kinase. We generated a mutant form of CK2alpha with V66A and I174A substitutions since previous reports demonstrated that replacement of V66 and I174 with less hydrophobic alanine residues rendered CK2alpha much less sensitive (>20-fold) to TBB and its derivatives [25,29]. Using this inhibitor-resistant mutant of CK2alpha, we demonstrated that phosphorylation of a CK2 substrate peptide (RRRDDDSDDD) [41] or immunoprecipitated EEF1D-FLAG could be rescued using either free catalytic subunit or CK2 holoenyzme [13]. Rescue of EEF1D phosphorylation, assessed using phospho-specific pSer162 EEF1D antibodies, was also demonstrated by expression of CK2alpha (V66A/I174A) together with EEFID-FLAG in HeLa cells. Overall, our results confirmed target engagement with the CK2 inhibitor and also validated that EEF1D was a *bona fide* physiological substrate of CK2.

While our strategy (Fig. 1) has been employed for the validation of CK2 inhibitors and the identification of a physiological CK2 substrate, it is versatile because it can be used in combination with other phosphoproteomic strategies (e.g. immobilized metal affinity chromatography (IMAC) or other phosphoprotein and phosphopeptide enrichment strategy prior to MS analysis, etc.) [42,43]. Furthermore, as an alternative to the utilization of inhibitor-resistant forms of individual protein kinases, analog-sensitive kinases that have been pioneered by Shokat and colleagues [44–46] can be employed for the identification of direct kinase:substrate relationships. Beyond their utility to validate the actions of kinase inhibitors in experimental systems, the identification of physiological substrates for protein kinases can also lead to the development of biomarkers that are necessary for tracking the actions of inhibitor that emerge as therapeutic agents.

3. Chemical proteomics using ATP-sepharose

A second key element in the validation of protein kinase inhibitors is an unbiased evaluation of their targets within complex mixtures that would be representative of potential off-targets. Given the high level of architectural relatedness between the >500 protein kinase encoded within the human genome, this issue is of particular concern for ATP-competitive kinase inhibitors [1,47]. Furthermore, since the purinome (i.e., proteins that bind ATP and/or GTP) [48], extends well beyond the kinome, it is critical to consider off-targets that may not be protein kinases. Again, while there is a vast literature describing protein kinase inhibitors, there is often rather limited information available